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U S DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

ATTORNEY'S DOCKET NUMBER: 8484-092-999

09/743205

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/DE99/02359	3 August 1999	3 August 1998

TITLE OF INVENTION

FIBROUS PROTEINS AND THE PRODUCTION THEREOF

APPLICANT(S) FOR DO/EO/US

Klaus Düring

Applicant herewith submits to the United States Designated/ Elected Office (DO/EO/US) the following items under 35 U.S.C. 371:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☒ is transmitted herewith (required only if not transmitted by the international Bureau).
   b. ☐ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)

6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureaus.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.

8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 37(c)(3)).

9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)) (unexecuted).

10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.

14. ☒ A substitute specification and marked-up version thereof comparing the substitute specification to the English translation of the priority application.

15. ☐ A change of power of attorney and/or address letter.

16. ☒ Other items or information:

Copies of:
- Request for International Application;
- First page of published PCT Application;
- International Search Report;
- Request for Preliminary Examination.

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INTERNATIONAL APPLICATION NO PCT/DE99/02359 09/743205	INTERNATIONAL FILING DATE 3 August 1999

528 Rec'd PCT/PTO 03 JAN 2001

17. ☒ The U.S. National Fee (35 U.S.C. 371(c)(1)) and other fees as follows:

CLAIMS				
(1)FOR	(2)NUMBER FILED	(3)NUMBER EXTRA	(4)RATE	(5)CALCULATIONS
TOTAL CLAIMS	47 - 20	27	X $ 18.00	$ 486.00
INDEPENDENT CLAIMS	4 - 3	1	X $ 80.00	80.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $ 270.00	$ 260.00
BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)): **CHECK ONE BOX ONLY** ☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) ..... $ 690				
☐ No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) ..... $ 710				
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO $1000				
☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2) to (4) ..... $ 100				
☒ Filing with EPO or JPO search report ..... $ 860				$ 860.00
Surcharge of $130.00 for furnishing the National fee or oath or declaration later than 20 30 mos. from the earliest claimed priority date (37 CFR 1.492(e)).				
			TOTAL OF ABOVE CALCULATIONS	= 1,686.00
Reduction by 1/2 for filing by small entity, if applicable. Affidavit must be filed also. (Note 37 CFR 1.9, 1.27, 1.28).				- $ 0.00
			SUBTOTAL	= 1,686.00
Processing fee of $130.00 for furnishing the English Translation later than 20 30 mos. from the earliest claimed priority date (37 CFR 1.492(f)).				+
0			TOTAL FEES ENCLOSED	$ 1,686.00

a. ☐ A check in the amount of $__ to cover the above fees is enclosed.

b. ☒ Please charge Deposit Account No. 16-1150 in the amount of $ 1686.00 to cover the above fees. A copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 16-1150 (order no. 8484-092-999). A copy of this sheet is enclosed.

18. ☐ Other instructions

n/a

19. ☒ All correspondence for this application should be mailed to

PENNIE & EDMONDS LLP
1155 AVENUE OF THE AMERICAS
NEW YORK, NEW YORK 10036-2711

20. ☒ All telephone inquiries should be made to (212) 790-2803

Birgit Millauer		43,341	January 3, 2001
NAME	SIGNATURE	REGISTRATION NUMBER	DATE

Laura A. Coruzzi
For: Laura A. Coruzzi
(Reg. No. 30,742)

Express Mail No.: EL 451 595 928 US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Düring *et al.*

Serial No.: To be assigned

Group Art Unit: To be assigned

Filed: Herewith

Examiner: To be assigned

For: **FIBROUS PROTEINS AND THE PRODUCTION THEREOF**

Attorney Docket No.: 8484-092-999

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

In accordance with Rule 111 of the Rules of Practice, 37 C.F.R. § 1.111, please consider and enter the following amendments and remarks.

AMENDMENTS

IN THE SPECIFICATION:

Please amend the specification as follows:

Please replace the specification as filed in PCT/EP99/04517 by the enclosed Substitute Specification under 37 C.F.R. § 1.125. The Substitute Specification has been prepared solely for the purpose of complying with the rules of practice; it does not introduce new matter. A marked-up copy of the Substitute Specification showing any matter being added and any matter being deleted from the original specification is enclosed in accordance with 37 C.F.R. § 1.125(b)(2).

IN THE CLAIMS:

Please amend the claims as follows:

1. (Amended) A process for the production of a fibrous protein, comprising [the following steps]:

(a) [expression of] expressing a precursor fibrous protein in a plant cell[,]; and

(b) [incubation of] incubating the precursor fibrous protein with a protein processing it.

2. (Amended) The process [according to claim] of Claim 1, wherein [the] said processing protein is expressed in a plant cell.

3. (Amended) The process [according to claim] of Claim 2, wherein [the] said precursor fibrous protein and the protein processing [it] said precursor protein are expressed in different plant cells.

4. (Amended) The process [according to claim] of Claim 2, wherein [the] said precursor fibrous protein and the protein processing [it] said precursor fibrous protein are expressed in the same plant cell.

5. (Amended) The process [according to any one of claims] of Claim 1 [to], 2, 3, or 4, wherein [the] said plant cell is available [in the form] as part of a plant.

6. (Amended) The process [according to any one of claims] of Claim 1 [to 5], 2, 3 or 4, wherein [the] said precursor fibrous protein is a procollagen or a derivative and fragment thereof[, respectively].

7. (Amended) The process [according to any one of claims] of Claim 1 [to 5], 2, 3, or 4, wherein [the] said precursor fibrous protein is a tropoelastin or a derivative and fragment thereof[, respectively].

8. (Amended) The process [according to any one of claims] of Claim 1 [to 6], 2, 3, or 4, wherein [the] said fibrous protein is a collagen or a derivative and fragment thereof[, respectively].

9. (Amended) The process [according to any one of claims] of Claim 1 [to 5 and 7], 2, 3, or 4, wherein [the] said fibrous protein is an elastin or a derivative and fragment thereof[, respectively].

10. (Amended) The process [according to any one of claims] of Claim 1 [to 9], 2, 3, or 4, wherein the protein processing precursor fibrous protein is a lysine oxidase.

11. (Amended) A plant cell[,] expressing a precursor fibrous protein and a protein processing [it] said precursor fibrous protein.

12. (Amended) The plant cell [according to claim] of Claim 11, wherein [the] said plant cell is [available in the form] part of a multiplication material.

13. (Amended) The plant cell [according to claim] of Claim 11, wherein [the] said plant cell is present [in the form] as part of a plant.

14. (Amended) [The] A plant cell[,] expressing a protein processing precursor fibrous protein.

15. (Amended) The plant cell [according to claim] of Claim 14, wherein [the] said plant cell is available [in the form] as part of a multiplication material.

16. (Amended) The plant cell [according to claim] of Claim 14, wherein [the] said plant cell is available [in the form] as part of a plant.

17. (Amended) [Use of the plant cell according to any one of claims 11 to 16] A method for the production of a fibrous protein, comprising expressing a precursor fibrous protein and a protein processing said precursor fibrous protein in a plant cell.

18. (Amended) [The] A fibrous protein[,] produced [according to] by the process [as defined in any one of claims] of Claim 1 [to 10] , 2, 3, or 4.

19. (Amended) The fibrous protein [according to claim] of Claim 18, wherein [the] said fibrous protein is a collagen or a derivative and fragment thereof[, respectively].

20. (Amended) The fibrous protein [according to claim] of Claim 18, wherein [the] said fibrous protein is an elastin or a derivative and fragment thereof[, respectively].

REMARKS

Claims 1-20 are pending in this application. The claims as pending are attached hereto as *Appendix A*.

The above amendments do not introduce new matter, and they are fully supported by the specification of the subject application and the claims as originally filed.

Applicants respectfully request that the above-made amendments be made of record in the file history of the instant application.

Respectfully submitted,

Date January 3, 2000

43,341
Birgit Millauer (Reg. No.)

For: Laura A. Coruzzi (Reg. No. 30,742)
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APPENDIX A
Claims as Pending
8484-092-999

1. A process for the production of a fibrous protein, comprising:

(a) expressing a precursor fibrous protein in a plant cell; and

(b) incubating the precursor fibrous protein with a protein processing it.

2. The process of Claim 1, wherein said processing protein is expressed in a plant cell.

3. The process of Claim 2, wherein said precursor fibrous protein and the protein processing said precursor protein are expressed in different plant cells.

4. The process of Claim 2, wherein said precursor fibrous protein and the protein processing said precursor fibrous protein are expressed in the same plant cell.

5. The process of Claim 1, 2, 3, or 4, wherein said plant cell is available as part of a plant.

6. The process of Claim 1, 2, 3 or 4, wherein said precursor fibrous protein is a procollagen or a derivative and fragment thereof.

7. The process of Claim 1, 2, 3, or 4, wherein said precursor fibrous protein is a tropoelastin or a derivative and fragment thereof.

8. The process of Claim 1, 2, 3, or 4, wherein said fibrous protein is a collagen or a derivative and fragment thereof.

9. The process of Claim 1, 2, 3, or 4, wherein said fibrous protein is an elastin or a derivative and fragment thereof.

10. The process of Claim 1, 2, 3, or 4, wherein the protein processing precursor fibrous protein is a lysine oxidase.

11. A plant cell expressing a precursor fibrous protein and a protein processing said precursor fibrous protein.

12. The plant cell of Claim 11, wherein said plant cell is part of a multiplication material.

13. The plant cell of Claim 11, wherein said plant cell is present as part of a plant.

14. A plant cell expressing a protein processing precursor fibrous protein.

15. The plant cell of Claim 14, wherein said plant cell is available as part of a multiplication material.

16. The plant cell of Claim 14, wherein said plant cell is available as part of a plant.

17. A method for the production of a fibrous protein, comprising expressing a precursor fibrous protein and a protein processing said precursor fibrous protein in a plant cell.

18. A fibrous protein produced by the process of Claim 1, 2, 3, or 4.

19. The fibrous protein of Claim 18, wherein said fibrous protein is a collagen or a derivative and fragment thereof.

20. The fibrous protein of Claim 18, wherein said fibrous protein is an elastin or a derivative and fragment thereof.

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

FIBROUS PROTEINS AND THEIR PRODUCTION

Inventor: **Klaus Düring**

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Attorney Docket No.: 8484-092-999

TABLE OF CONTENTS

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

FIBROUS PROTEINS AND THEIR PRODUCTION

This is a national phase filing of the Application No. PCT/DE99/02359, which was filed with the Patent Corporation Treaty on 3 August 1999, and is entitled to priority of the German Patent Application 198 34 909.2, filed 3 August 1998.

I. FIELD OF THE INVENTION

The present invention relates to a process for the production of fibrous proteins in plant cells, plant cells usable for this purpose and fibrous proteins obtained by the process.

II. BACKGROUND OF THE INVENTION

Fibrous proteins are proteins having mechanical stability, *e.g.*, resilience or elasticity. They form from precursor fibrous proteins which are polymerized and cross-linked, respectively. This requires the presence of repetitive amino acid sequences in the precursor fibrous proteins and the influence of proteins which process precursor fibrous proteins. Fibrous proteins are found in animal and human cells. Examples of fibrous proteins are collagen and elastin. Both are components of connective tissues, *e.g.*, skin, tendons, ligaments and blood vessels. Collagen forms by cross-linkage of tropocollagen molecules, while elastin is formed by cross-linkage of tropoelastin molecules.

Fibrous proteins are used for medical purposes and cosmetic purposes, respectively. To this end, they are frequently isolated from animal cells. This involves a great risk, since animal diseases, *e.g.*, BSE, can be transmitted to man in this way.

Therefore, it is the object of the present invention to provide a process by which fibrous proteins can be produced without the above risks.

According to the invention this is achieved by the subject matters defined in the claims.

III. SUMMARY OF THE INVENTION

The present invention relates to a process for the production of a fibrous protein, comprising the following steps:

(a) expression of a precursor fibrous protein in a plant cell, and

(b) incubation of the precursor fibrous protein with a protein processing it.

Furthermore, this invention concerns plant cells usable for this purpose and fibrous proteins obtained by this process.

IV. DETAILED DESCRIPTION OF THE INVENTION

It is the object of the present invention to provide a process by which fibrous proteins can be produced without the above risks.

According to the invention this is achieved by the subject matters defined in the claims.

The present invention is based on the applicant's findings that precursor fibrous proteins can be produced in plant cells, which can then be converted into the corresponding fibrous proteins by treatment with proteins processing them. In particular, he found that precursor fibrous proteins can be produced in both individual plant cells and plants. He also discovered that the conversion of precursor fibrous proteins into the corresponding fibrous proteins can be made *in vitro* and *in vivo*. In the latter case, this can be made, *e.g.*, in that the precursor fibrous protein is expressed in a plant cell together with the protein processing it. The applicant made his discoveries using individual plant cells and plants, particularly the potato plant.

According to the invention the applicant's findings are use for a process for the production of a fibrous protein, which comprises the following steps:

(a) expression of a precursor fibrous protein in a plant cell, and

(b) incubation of the precursor fibrous protein with a protein processing it.

The expression "fibrous protein" comprises a fibrous protein of any kind and origin. It may have two-dimensional or three-dimensional cross-linked structure. It can also be an animal or human fibrous protein. In addition, it may be available in wild-type or modified form. The latter comprises a fibrous protein whose amino acid sequence is modified as compared to the wild-type or modified form. The latter comprises a fibrous protein whose amino acid sequence is modified as compared to the wild-type sequence at one or more sites. Such modifications may be additions, substitutions, deletions and/or inversions of one or more amino acids. In particular, amino acids may be present which are preferably expressed in plant cells. Besides, the fibrous protein may be a fusion protein, the fusion partner being, *e.g.*, oleosin. This protein then enables the localization of the fibrous protein in the oil phase

of vegetable multiplication material. Fibrous proteins which are available in modified form have mechanical stability, *e.g.*, resilience or elasticity, which is at least comparable to that of the wild-type form. Preferred fibrous proteins are collagen and elastin as well as derivatives and fragments thereof, respectively. As regards a modified form, the above statements apply to them correspondingly.

The term "expression of a precursor fibrous protein" comprises any expression of a gene coding for a precursor fibrous protein in a plant cell, the precursor fibrous protein being convertible into the corresponding fibrous protein as usual, *e.g.*, by cross-linkage or polymerization. The above statements made on the expression "fibrous protein" apply here correspondingly. In addition, the precursor fibrous protein can be present with or without signal peptide. The former may be, *e.g.*, the natural or a foreign signal peptide, so that an extracellular localization of the precursor fibrous protein is enabled. In the latter, however, localization of the precursor fibrous protein is achieved in the cytoplasm. In addition, the precursor fibrous protein may have a control peptide so as to enable localization of the precursor fibrous protein in certain compartments of the plant cell, *e.g.*, ER, chloroplasts or vacuoles. Preferred precursor fibrous proteins are tropocollagen and tropoelastin as well as derivatives and fragments thereof, respectively. For the expression of a gene coding for a precursor fibrous protein it is possible to use conventional expression vectors for plant cells. They comprise regulatory elements, *e.g.*, enhancer, promoter and termination sequences detected in plant cells. Examples thereof are CaMV 35S promoter and termination sequences (Odell *et al.*, 1985, *Nature* 313:810-812). The expression vectors may also contain selection markers, *e.g.*, a neomycin or kanamycin resistance gene. In addition, the expression vectors may contain sequences which favor their introduction into plant cells. For example, the expression vectors may contain T-DNA of binary vectors, such as pSR 8-30 or pSR 8-35/1, when they shall be introduced into plants *via* Agrobacterium tumefaciens (Düring *et al.*, 1993, *Plant Journal* 3:587-598; Porsch *et al.*, 1998, *Plant Molecular Biology* 37:581-585). Besides, the expression vectors can also be introduced into plant cells by means of processes for which they do not require any special sequences. Such processes are, *e.g.*, microinjection, electroporation, DNA transfer by means of polyethylene glycol, liposome fusion or particle gun.

The expression "plant cell" comprises plant cells of any kind and origin. It may refer to individual plant cells, freshly isolated or established as a cell line, or those present in an

aggregation. The latter is, *e.g.*, a plant or part thereof. Examples of plants are monocotyl plants, such as corn, rice, wheat, barley and sugarcane, and dicotyl plants, such as potato, tobacco, tomato, tea, coffee, brassicaceaen, particularly rape and cabbage, and leguminaes, particularly pea, phaseolus, vicia and soybean.

The expression "protein processing precursor fibrous protein" comprises any protein which can convert a precursor fibrous protein into the corresponding fibrous protein. The conversion can be made as usual, *e.g.*, by cross-linkage or polymerization. Examples of such a protein are lysine oxidases. Also, proteinases may be concerned which, *e.g.*, in the case of collagen, have been described. The lysine oxidases and proteinases, respectively, may be present as such and as derivatives or fragments thereof, respectively. The above statements made on a modified form of a fibrous protein apply correspondingly to them.

The expression "incubation of a precursor fibrous protein with a protein processing it" comprises any incubation of these proteins by which the precursor fibrous protein can be converted into the corresponding fibrous protein. The incubation may be made, *e.g.*, *in vitro*. For this purpose, it is favorable to incubate the expressed precursor fibrous protein in solution with the protein processing it. The incubation can also be carried out *in vivo*. For this purpose, it is favorable to express not only the precursor fibrous protein but also the protein processing it in a plant cell. Both proteins can be expressed in different plant cells which are then combined whereby the precursor fibrous protein is incubated with the protein processing it. The precursor fibrous protein and the protein processing it can also be expressed in the same plant cell. Thus, both proteins are automatically incubated in this plant cell. The above statements made on the expression of a precursor fibrous protein apply correspondingly to the expression of a protein processing a precursor fibrous protein.

A further subject matter of the present invention relates to a plant cell which expresses a precursor fibrous protein and a protein processing it. Also, a plant cell is preferred which expresses only the latter of these proteins. Regarding the expressions "plant cell", "precursor fibrous protein" and "protein processing precursor fibrous protein" reference is made to the above statements. In addition, the plant cell may be available in the form of a multiplication material.

Common methods can be used for the production of a plant cell according to the invention. In supplement to the above statements, the production of a plant according to the invention which expresses a precursor fibrous protein, *e.g.*, tropoelastin, and a protein

processing it, *e.g.*, lysine oxidase, is described by way of example. In this connection, it is favorable to provide a cDNA coding for tropoelastin with CaNV 35S promoter and termination sequences and insert it in a binary vector, *e.g.*, pSR 8-30 and pSR 8-35/1, respectively. The same can be done with a cDNA coding for a lysine oxidase. The resulting DNA molecules are used for transforming bacteria, *e.g., E. coli* S17-1 which are suitable for a transfer of the DNA molecules to *Agrobacterium tumefaciens, e.g.*, CV 3101. For this purpose, *E. coli* S17-1 and *Agrobacterium tumefaciens* GV 3101 are mixed with each other and incubated overnight. Agrobacteria which have taken up the DNA molecules are selected by growth on carbenicillin containing medium. They are then applied to cut-off potato plant leaves whose middle ribs were scratched several times and incubated in the dark for two days. Thereafter, the agrobacteria are removed and growth promoters are added to the potato plants, so that sprouts grow. They are cut off and used for cultivating new potato plants. The detection of the expression products tropoelastin and lysine oxidase and/or the resulting elastin is made by means of specific antibodies against these proteins. Reference is made to the below examples.

By means of the present invention it is possible to produce fibrous proteins in plant cells, particularly plants, in high purity. Therefore, the fibrous proteins are suitable for the most varying applications. They are found *e.g.*, in agriculture, chemistry, production of cosmetics and medicine. In the latter case, *e.g.*, the use of fibrous proteins for transplants and wound closures has to be mentioned. In particular, the fibrous proteins distinguish themselves in that they are free from animal or human viruses and pathogens, respectively. Moreover, the fibrous proteins can be produced in huge amounts. This applies particularly when they are isolated from plants cultivated in fields. Thus, the present invention represents a great contribution to providing pharmaceutical preparations safely and in great amounts.

The below examples explain the invention in more detail. The following preparations and examples are given to enable those skilled in the art to more clearly understand and to practice the present invention. The present invention, however, is not limited in scope by the exemplified embodiments, which are intended as illustrations of single aspects of the invention only, and methods which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and

accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

V. EXAMPLES

A. Example 1: Production Of Elastin In Potato Plants

A cDNA is used for human elastin (Fazio, 1988, *Journal of Investigative Dermatology* 91:458-464). This cDNA is provided with an NcoI restriction site at its 5' end and with an XbaI restriction site at its 3' end by means of PCR. The resulting cDNA fragment is inserted in the vector pRT 100 which contains an expression cassette having CaNV 35S promoter and termination sequences (Töpfer *et al.*, 1987, *Nucleic Acids Research* 15:5890; Odell *et al.*, *supra*). Following cleavage using HindIII, the expression cassette containing the elastin cDNA is isolated and inserted in the binary vector pSR 8-30 (Düring *et al.*, *supra*; Porsch *et al.*, *supra*). The expression vector pSR 8-30 elastin is obtained.

In addition, a cDNA for human lysine oxidase is used (Hämäläinen, 1991, *Genomics* 11:508-516). It is treated as described above and inserted in the binary vector pSR 8-30. The expression vector pSR 8-30 lysine oxidase is obtained.

The expression vectors pSR 8-30 elastin and pSR 8-30 lysine oxidase are used for transforming E. coli S17-1. The transformants are mixed with *Agrobacterium tumefaciens* CV 3101 and incubated at 27°C overnight (Koncz and Shell, 1986, *Molecular and General Genetics* 204:383-396; Koncz *et al.*, 1987, *Proc. Natl. Acad. Sci. U.S.A.* 84:131-135). Selection on carbenicillin is carried out, the bla gene necessary for this purpose being present in the above expression vectors. Selection clones of *Agrobacterium tumefaciens* are applied to cut-off leaves of potato plant cv. or named Désirée, whose middle ribs had been scratched several times and the plant is incubated in the dark at 20°C for 2 days. Thereafter, the agrobacteria are separated and growth promoters are added to the potato plant, so that sprouts form preferably. Moreover, non-transformed cells of the potato plant are killed by the addition of kanamycin to the plant medium. Rising sprouts are cut off and are allowed to grow roots on medium without plant growth substances but with kanamycin. The potato plants are further cultivated as usual.

The analysis of the expressed tropoelastin and lysine oxidase and/or the resulting elastin is achieved by antibodies in Western blot and ELISA, respectively, which are specific

to the individual proteins. For this purpose, whole protein or the intercellular wash liquid of the potato plant is isolated and used in the corresponding detection methods.

It shows that tropoelastin and lysine oxidase can be expressed in plant cells, particularly in a plant. Moreover, it shows that by the incubation of lysine oxidase with the tropoelastin the latter is converted into elastin which can be isolated in pure form.

B. Example 2: Production Of Collagen In Potato Plants

cDNAs are used which code for the subunits α1 and α2 of human tropocollagen (Chu *et al.*, 1985, *Journal of Biological Chemistry* 260:2315-2320; Dickson *et al.*, 1985, *Nucleic Acids Res.* 13:3427-3438). Furthermore, cDNAs are used which code for human lysine oxidase, human procollagen C proteinase and procollagen N proteinase, respectively, from bovine animals (Hämäläinen *et al.*, *supra*; Li *et al.*, 1996, *Proc. Natl. Acad. Sci U.S.A.* 93:5127-5130; Colige *et al.*, 1997, *Proc. Natl. Acad. Sci. U.S.A.* 94:2374-2379)

These DNAs are treated as described in Example 1 and inserted in the pSR 8-30 vector. The expression vectors pSR 8-30 tropocollagen α1, pSR 8-30 tropocollagen α2, pSR 8-30 lysine oxidase, pSR 8-30 C proteinase and pSR 8-30 N proteinase are obtained. The procedure is continued as described in Example 1.

It shows that tropocollagen and proteins processing it can be expressed in plant cells, particularly in a plant. In addition, it shows that collagen having a high degree of purity can be obtained.

All references cited within the body of the instant specification are hereby incorporated by reference in their entirety.

CLAIMS

WHAT IS CLAIMED:

1. A process for the production of a fibrous protein, comprising the following steps:

(a) expression of a precursor fibrous protein in a plant cell, and

(b) incubation of the precursor fibrous protein with a protein processing it.

2. The process according to claim 1, wherein the processing protein is expressed in a plant cell.

3. The process according to claim 2, wherein the precursor fibrous protein and the protein processing it are expressed in different plant cells.

4. The process according to claim 2, wherein the precursor fibrous protein and the protein processing it are expressed in the same plant cell.

5. The process according to any one of claims 1 to 4, wherein the plant cell is available in the form of a plant.

6. The process according to any one of claims 1 to 5, wherein the precursor fibrous protein is a procollagen or a derivative and fragment thereof, respectively.

7. The process according to any one of claims 1 to 5, wherein the precursor fibrous protein is a tropoelastin or a derivative and fragment thereof, respectively.

8. The process according to any one of claims 1 to 6, wherein the fibrous protein is a collagen or a derivative and fragment thereof, respectively.

9. The process according to any one of claims 1 to 5 and 7, wherein the fibrous protein is an elastin or a derivative and fragment thereof, respectively.

10. The process according to any one of claims 1 to 9, wherein the protein processing precursor fibrous protein is a lysine oxidase.

11. A plant cell, expressing a precursor fibrous protein and a protein processing it.

12. The plant cell according to claim 11, wherein the plant cell is available in the form of a multiplication material.

13. The plant cell according to claim 11, wherein the plant cell is present in the form of a plant.

14. The plant cell, expressing a protein processing precursor fibrous protein.

15. The plant cell according to claim 14, wherein the plant cell is available in the form of a multiplication material.

16. The plant cell according to claim 14, wherein the plant cell is available in the form of a plant.

17. Use of the plant cell according to any one of claims 11 to 16 for the production of a fibrous protein.

18. The fibrous protein, produced according to the process as defined in any one of claims 1 to 10.

19. The fibrous protein according to claim 18, wherein the fibrous protein is a collagen or a derivative and fragment thereof, respectively.

20. The fibrous protein according to claim 18, wherein the fibrous protein is an elastin or a derivative and fragment thereof, respectively.

ABSTRACT

The present invention relates to a process for the production of a fibrous protein, comprising the following steps:

(a) expression of a precursor fibrous protein in a plant cell, and

(b) incubation of the precursor fibrous protein with a protein processing it.

Furthermore, this invention concerns plant cells usable for this purpose and fibrous proteins obtained by this process.

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

FIBROUS PROTEINS AND THEIR PRODUCTION

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TABLE OF CONTENTS

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

FIBROUS PROTEINS AND THEIR PRODUCTION

This is a national phase filing of the Application No. PCT/DE99/02359, which was filed with the Patent Corporation Treaty on 3 August 1999, and is entitled to priority of the German Patent Application 198 34 909.2, filed 3 August 1998.

I. FIELD OF THE INVENTION [Fibrous Proteins and Their Production]

The present invention relates to a process for the production of fibrous proteins in plant cells, plant cells usable for this purpose and fibrous proteins obtained by the process.

II. BACKGROUND OF THE INVENTION

Fibrous proteins are proteins having mechanical stability, *e.g.*, resilience or elasticity. They form from precursor fibrous proteins which are polymerized and cross-linked, respectively. This requires the presence of repetitive amino acid sequences in the precursor fibrous proteins and the influence of proteins which process precursor fibrous proteins. Fibrous proteins are found in animal and human cells. Examples of fibrous proteins are collagen and elastin. Both are components of connective tissues, *e.g.*, skin, tendons, ligaments and blood vessels. Collagen forms by cross-linkage of tropocollagen molecules, while elastin is formed by cross-linkage of tropoelastin molecules.

Fibrous proteins are used for medical purposes and cosmetic purposes, respectively. To this end, they are frequently isolated from animal cells. This involves a great risk, since animal diseases, *e.g.*, BSE, can be transmitted to man in this way.

Therefore, it is the object of the present invention to provide a process by which fibrous proteins can be produced without the above risks.

According to the invention this is achieved by the subject matters defined in the claims.

III. SUMMARY OF THE INVENTION

The present invention relates to a process for the production of a fibrous protein, comprising the following steps:

(a) expression of a precursor fibrous protein in a plant cell, and

(b) incubation of the precursor fibrous protein with a protein processing it.

Furthermore, this invention concerns plant cells usable for this purpose and fibrous proteins obtained by this process.

IV. DETAILED DESCRIPTION OF THE INVENTION

It is the object of the present invention to provide a process by which fibrous proteins can be produced without the above risks.

According to the invention this is achieved by the subject matters defined in the claims.

The present invention is based on the applicant's findings that precursor fibrous proteins can be produced in plant cells, which can then be converted into the corresponding fibrous proteins by treatment with proteins processing them. In particular, he found that precursor fibrous proteins can be produced in both individual plant cells and plants. He also discovered that the conversion of precursor fibrous proteins into the corresponding fibrous proteins can be made *in vitro* and *in vivo*. In the latter case, this can be made, *e.g.*, in that the precursor fibrous protein is expressed in a plant cell together with the protein processing it. The applicant made his discoveries using individual plant cells and plants, particularly the potato plant.

According to the invention the applicant's findings are use for a process for the production of a fibrous protein, which comprises the following steps:

(a) expression of a precursor fibrous protein in a plant cell, and

(b) incubation of the precursor fibrous protein with a protein processing it.

The expression "fibrous protein" comprises a fibrous protein of any kind and origin. It may have two-dimensional or three-dimensional cross-linked structure. It can also be an animal or human fibrous protein. In addition, it may be available in wild-type or modified form. The latter comprises a fibrous protein whose amino acid sequence is modified as compared to the wild-type or modified form. The latter comprises a fibrous protein whose amino acid sequence is modified as compared to the wild-type sequence at one or more sites. Such modifications may be additions, substitutions, deletions and/or inversions of one or more amino acids. In particular, amino acids may be present which are preferably expressed in plant cells. Besides, the fibrous protein may be a fusion protein, the fusion partner being, *e.g.*, oleosin. This protein then enables the localization of the fibrous protein in the oil phase

of vegetable multiplication material. Fibrous proteins which are available in modified form have mechanical stability, *e.g.*, resilience or elasticity, which is at least comparable to that of the wild-type form. Preferred fibrous proteins are collagen and elastin as well as derivatives and fragments thereof, respectively. As regards a modified form, the above statements apply to them correspondingly.

The term "expression of a precursor fibrous protein" comprises any expression of a gene coding for a precursor fibrous protein in a plant cell, the precursor fibrous protein being convertible into the corresponding fibrous protein as usual, *e.g.*, by cross-linkage or polymerization. The above statements made on the expression "fibrous protein" apply here correspondingly. In addition, the precursor fibrous protein can be present with or without signal peptide. The former may be, *e.g.*, the natural or a foreign signal peptide, so that an extracellular localization of the precursor fibrous protein is enabled. In the latter, however, localization of the precursor fibrous protein is achieved in the cytoplasm. In addition, the precursor fibrous protein may have a control peptide so as to enable localization of the precursor fibrous protein in certain compartments of the plant cell, *e.g.*, ER, chloroplasts or vacuoles. Preferred precursor fibrous proteins are tropocollagen and tropoelastin as well as derivatives and fragments thereof, respectively. For the expression of a gene coding for a precursor fibrous protein it is possible to use conventional expression vectors for plant cells. They comprise regulatory elements, *e.g.*, enhancer, promoter and termination sequences detected in plant cells. Examples thereof are CaMV 35S promoter and termination sequences [(cf. Odell, J.T.](Odell *et al.*, 1985, *Nature* 313[(1985),]:810-812). The expression vectors may also contain selection markers, *e.g.*, a neomycin or kanamycin resistance gene. In addition, the expression vectors may contain sequences which favor their introduction into plant cells. For example, the expression vectors may contain T-DNA of binary vectors, such as pSR 8-30 or pSR 8-35/1, when they shall be introduced into plants *via* Agrobacterium tumefaciens [(cf. Düring, K.](Düring *et al.*, 1993, *Plant Journal* 3[(1993),]:587-598; Porsch[, P.] *et al.*, 1998, *Plant Molecular Biology* 37[(1998),]:581-585). Besides, the expression vectors can also be introduced into plant cells by means of processes for which they do not require any special sequences. Such processes are, *e.g.*, microinjection, electroporation, DNA transfer by means of polyethylene glycol, liposome fusion or particle gun.

The expression "plant cell" comprises plant cells of any kind and origin. It may refer to individual plant cells, freshly isolated or established as a cell line, or those present in an

aggregation. The latter is, *e.g.*, a plant or part thereof. Examples of plants are monocotyl plants, such as corn, rice, wheat, barley and sugarcane, and dicotyl plants, such as potato, tobacco, tomato, tea, coffee, brassicaceaen, particularly rape and cabbage, and leguminaes, particularly pea, phaseolus, vicia and soybean.

The expression "protein processing precursor fibrous protein" comprises any protein which can convert a precursor fibrous protein into the corresponding fibrous protein. The conversion can be made as usual, *e.g.*, by cross-linkage or polymerization. Examples of such a protein are lysine oxidases. Also, proteinases may be concerned which, *e.g.*, in the case of collagen, have been described. The lysine oxidases and proteinases, respectively, may be present as such and as derivatives or fragments thereof, respectively. The above statements made on a modified form of a fibrous protein apply correspondingly to them.

The expression "incubation of a precursor fibrous protein with a protein processing it" comprises any incubation of these proteins by which the precursor fibrous protein can be converted into the corresponding fibrous protein. The incubation may be made, *e.g.*, *in vitro*. For this purpose, it is favorable to incubate the expressed precursor fibrous protein in solution with the protein processing it. The incubation can also be carried out *in vivo*. For this purpose, it is favorable to express not only the precursor fibrous protein but also the protein processing it in a plant cell. Both proteins can be expressed in different plant cells which are then combined whereby the precursor fibrous protein is incubated with the protein processing it. The precursor fibrous protein and the protein processing it can also be expressed in the same plant cell. Thus, both proteins are automatically incubated in this plant cell. The above statements made on the expression of a precursor fibrous protein apply correspondingly to the expression of a protein processing a precursor fibrous protein.

A further subject matter of the present invention relates to a plant cell which expresses a precursor fibrous protein and a protein processing it. Also, a plant cell is preferred which expresses only the latter of these proteins. Regarding the expressions "plant cell", "precursor fibrous protein" and "protein processing precursor fibrous protein" reference is made to the above statements. In addition, the plant cell may be available in the form of a multiplication material.

Common methods can be used for the production of a plant cell according to the invention. In supplement to the above statements, the production of a plant according to the invention which expresses a precursor fibrous protein, *e.g.*, tropoelastin, and a protein

processing it, *e.g.*, lysine oxidase, is described by way of example. In this connection, it is favorable to provide a cDNA coding for tropoelastin with CaNV 35S promoter and termination sequences and insert it in a binary vector, *e.g.*, pSR 8-30 and pSR 8-35/1, respectively. The same can be done with a cDNA coding for a lysine oxidase. The resulting DNA molecules are used for transforming bacteria, *e.g.*, *E. [coil] coli* S17-1 which are suitable for a transfer of the DNA molecules to *Agrobacterium tumefaciens, e.g.*, CV 3101. For this purpose, *E. coli* S17-1 and *Agrobacterium tumefaciens* GV 3101 are mixed with each other and incubated overnight. Agrobacteria which have taken up the DNA molecules are selected by growth on carbenicillin containing medium. They are then applied to cut-off potato plant leaves whose middle ribs were scratched several times and incubated in the dark for two days. Thereafter, the agrobacteria are removed and growth promoters are added to the potato plants, so that sprouts grow. They are cut off and used for cultivating new potato plants. The detection of the expression products tropoelastin and lysine oxidase and/or the resulting elastin is made by means of specific antibodies against these proteins. Reference is made to the below examples.

By means of the present invention it is possible to produce fibrous proteins in plant cells, particularly plants, in high purity. Therefore, the fibrous proteins are suitable for the most varying applications. They are found *e.g.*, in agriculture, chemistry, production of cosmetics and medicine. In the latter case, *e.g.*, the use of fibrous proteins for transplants and wound closures has to be mentioned. In particular, the fibrous proteins distinguish themselves in that they are free from animal or human viruses and pathogens, respectively. Moreover, the fibrous proteins can be produced in huge amounts. This applies particularly when they are isolated from plants cultivated in fields. Thus, the present invention represents a great contribution to providing pharmaceutical preparations safely and in great amounts.

The [invention is explained by the below examples] below examples explain the invention in more detail. The following preparations and examples are given to enable those skilled in the art to more clearly understand and to practice the present invention. The present invention, however, is not limited in scope by the exemplified embodiments, which are intended as illustrations of single aspects of the invention only, and methods which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in

the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

V. EXAMPLES

A. Example 1: Production Of Elastin In Potato Plants

A cDNA is used for human elastin [(cf. Fazio, M.J.](Fazio, 1988, *Journal of Investigative Dermatology* 91[(1988),]:458-464). This cDNA is provided with an NcoI restriction site at its 5' end and with an XbaI restriction site at its 3' end by means of PCR. The resulting cDNA fragment is inserted in the vector pRT 100 which contains an expression cassette having CaNV 35S promoter and termination sequences [(cf. Töpfer, R.](Töpfer *et al.*, 1987, *Nucleic Acids Research* 15[(1987),]:5890; Odell[, J.T.] *et al.*, *[above)] supra*). Following cleavage using HindIII, the expression cassette containing the elastin cDNA is isolated and inserted in the binary vector pSR 8-30 [(cf. Düring, K.](Düring *et al., supra*; Porsch[, P.] *et al.*, *[above)] supra*). The expression vector pSR 8-30 elastin is obtained.

In addition, a cDNA for human lysine oxidase is used [(cf. Hämäläinen, E.R.,](Hämäläinen, 1991, *Genomics* 11[(1991),]:508-516). It is treated as described above and inserted in the binary vector pSR 8-30. The expression vector pSR 8-30 lysine oxidase is obtained.

The expression vectors pSR 8-30 elastin and pSR 8-30 lysine oxidase are used for transforming E. coli S17-1. The transformants are mixed with *Agrobacterium tumefaciens* CV 3101 and incubated at 27°C overnight [(cf. Koncz, C.,](Koncz and Shell, [J.] 1986, *Molecular and General Genetics* 204[(1986),]:383-396; Koncz[, C.] *et al.*, 1987, *Proc. Natl. Acad. Sci. U.S.A.* 84[(1987),]:131-135). Selection on carbenicillin is carried out, the bla gene necessary for this purpose being present in the above expression vectors. Selection clones of *Agrobacterium tumefaciens* are applied to cut-off leaves of potato plant cv. or named Désirée, whose middle ribs had been scratched several times and the plant is incubated in the dark at 20°C for 2 days. Thereafter, the agrobacteria are separated and growth promoters are added to the potato plant, so that sprouts form preferably. Moreover, non-transformed cells of the potato plant are killed by the addition of kanamycin to the plant medium. Rising sprouts are cut off and are allowed to grow roots on medium without plant growth substances but with kanamycin. The potato plants are further cultivated as usual.

The analysis of the expressed tropoelastin and lysine oxidase and/or the resulting elastin is achieved by antibodies in Western blot and ELISA, respectively, which are specific to the individual proteins. For this purpose, whole protein or the intercellular wash liquid of the potato plant is isolated and used in the corresponding detection methods.

It shows that tropoelastin and lysine oxidase can be expressed in plant cells, particularly in a plant. Moreover, it shows that by the incubation of lysine oxidase with the tropoelastin the latter is converted into elastin which can be isolated in pure form.

B. Example 2: Production Of Collagen In Potato Plants

cDNAs are used which code for the subunits α1 and α2 of human tropocollagen [(cf. Chu, M.L.](Chu *et al.*, 1985, *Journal of Biological Chemistry* 260[(1985),]:2315-2320; Dickson *[L.A.] et al.*, 1985, *Nucleic Acids Res.* 13[(1985),]:3427-3438). Furthermore, cDNAs are used which code for human lysine oxidase, human procollagen C proteinase and procollagen N proteinase, respectively, from bovine animals [(cf. Hämäläinen, E.R.](Hämäläinen *et al.*, *[above;] supra;* Li[, S.W.] *et al.*, 1996, *Proc. Natl. Acad. Sci U.S.A.* 93[(1996),]:5127-5130; Colige[, A.] *et al.*, 1997, *Proc. Natl. Acad. [Sd] Sci. U.S.A.* 94[(1997),]:2374-2379)

These DNAs are treated as described in Example 1 and inserted in the pSR 8-30 vector. The expression vectors pSR 8-30 tropocollagen α1, pSR 8-30 tropocollagen α2, pSR 8-30 lysine oxidase, pSR 8-30 C proteinase and pSR 8-30 N proteinase are obtained. The procedure is continued as described in Example 1.

It shows that tropocollagen and proteins processing it can be expressed in plant cells, particularly in a plant. In addition, it shows that collagen having a high degree of purity can be obtained.

All references cited within the body of the instant specification are hereby incorporated by reference in their entirety.

CLAIMS

WHAT IS CLAIMED: [Claims]

1. A process for the production of a fibrous protein, comprising the following steps:

(a) expression of a precursor fibrous protein in a plant cell, and

(b) incubation of the precursor fibrous protein with a protein processing it.

2. The process according to claim 1, wherein the processing protein is expressed in a plant cell.

3. The process according to claim 2, wherein the precursor fibrous protein and the protein processing it are expressed in different plant cells.

4. The process according to claim 2, wherein the precursor fibrous protein and the protein processing it are expressed in the same plant cell.

5. The process according to any one of claims 1 to 4, wherein the plant cell is available in the form of a plant.

6. The process according to any one of claims 1 to 5, wherein the precursor fibrous protein is a procollagen or a derivative and fragment thereof, respectively.

7. The process according to any one of claims 1 to 5, wherein the precursor fibrous protein is a tropoelastin or a derivative and fragment thereof, respectively.

8. The process according to any one of claims 1 to 6, wherein the fibrous protein is a collagen or a derivative and fragment thereof, respectively.

9. The process according to any one of claims 1 to 5 and 7, wherein the fibrous protein is an elastin or a derivative and fragment thereof, respectively.

10. The process according to any one of claims 1 to 9, wherein the protein processing precursor fibrous protein is a lysine oxidase.

11. A plant cell, expressing a precursor fibrous protein and a protein processing it.

12. The plant cell according to claim 11, wherein the plant cell is available in the form of a multiplication material.

13. The plant cell according to claim 11, wherein the plant cell is present in the form of a plant.

14. The plant cell, expressing a protein processing precursor fibrous protein.

15. The plant cell according to claim 14, wherein the plant cell is available in the form of a multiplication material.

16. The plant cell according to claim 14, wherein the plant cell is available in the form of a plant.

17. Use of the plant cell according to any one of claims 11 to 16 for the production of a fibrous protein.

18. The fibrous protein, produced according to the process as defined in any one of claims 1 to 10.

19. The fibrous protein according to claim 18, wherein the fibrous protein is a collagen or a derivative and fragment thereof, respectively.

20. The fibrous protein according to claim 18, wherein the fibrous protein is an elastin or a derivative and fragment thereof, respectively.

[Abstract of the Disclosure]

ABSTRACT

The present invention relates to a process for the production of a fibrous protein, comprising the following steps:

(a) expression of a precursor fibrous protein in a plant cell, and

(b) incubation of the precursor fibrous protein with a protein processing it.

Furthermore, this invention concerns plant cells usable for this purpose and fibrous proteins obtained by this process.

Fibrous Proteins and Their Production

The present invention relates to a process for the production of fibrous proteins in plant cells, plant cells usable for this purpose and fibrous proteins obtained by the process.

Fibrous proteins are proteins having mechanical stability, e.g. resilience or elasticity. They form from precursor fibrous proteins which are polymerized and cross-linked, respectively. This requires the presence of repetitive amino acid sequences in the precursor fibrous proteins and the influence of proteins which process precursor fibrous proteins. Fibrous proteins are found in animal and human cells. Examples of fibrous proteins are collagen and elastin. Both are components of connective tissues, e.g. skin, tendons, ligaments and blood vessels. Collagen forms by cross-linkage of tropocollagen molecules, while elastin is formed by cross-linkage of tropoelastin molecules.

Fibrous proteins are used for medical purposes and cosmetic purposes, respectively. To this end, they are frequently isolated from animal cells. This involves a great risk, since animal diseases, e.g. BSE, can be transmitted to man in this way.

Therefore, it is the object of the present invention to provide a process by which fibrous proteins can be produced without the above risks.

According to the invention this is achieved by the subject matters defined in the claims.

The present invention is based on the applicant's findings that precursor fibrous proteins can be produced in plant cells, which can then be converted into the corresponding fibrous proteins by treatment with proteins processing them. In particular, he found that precursor fibrous proteins can be produced in both individual plant cells and plants. He also discovered that the conversion of precursor fibrous

proteins into the corresponding fibrous proteins can be made *in vitro* and *in vivo*. In the latter case, this can be made e.g. in that the precursor fibrous protein is expressed in a plant cell together with the protein processing it. The applicant made his discoveries using individual plant cells and plants, particularly the potato plant.

According to the invention the applicant's findings are used for a process for the production of a fibrous protein, which comprises the following steps:

(a) expression of a precursor fibrous protein in a plant cell, and

(b) incubation of the precursor fibrous protein with a protein processing it.

The expression "fibrous protein" comprises a fibrous protein of any kind and origin. It may have a two-dimensional or three-dimensional cross-linked structure. It can also be an animal or human fibrous protein. In addition, it may be available in wild-type or modified form. The latter comprises a fibrous protein whose amino acid sequence is modified as compared to the wild-type sequence at one or more sites. Such modifications may be additions, substitutions, deletions and/or inversions of one or more amino acids. In particular, amino acids may be present which are preferably expressed in plant cells. Besides, the fibrous protein may be a fusion protein, the fusion partner being e.g. oleosin. This protein then enables the localization of the fibrous protein in the oil phase of vegetable multiplication material. Fibrous proteins which are available in modified form have mechanical stability, e.g. resilience or elasticity, which is at least comparable to that of the wild-type form. Preferred fibrous proteins are collagen and elastin as well as derivatives and fragments thereof, respectively. As regards a modified form, the above statements apply to them correspondingly.

The term "expression of a precursor fibrous protein" comprises any expression of a gene coding for a precursor fibrous protein in a plant cell, the precursor fibrous

protein being convertible into the corresponding fibrous protein as usual, e.g. by cross-linkage or polymerization. The above statements made on the expression "fibrous protein" apply here correspondingly. In addition, the precursor fibrous protein can be present with or without signal peptide. The former may be e.g. the natural or a foreign signal peptide, so that an extracellular localization of the precursor fibrous protein is enabled. In the latter, however, localization of the precursor fibrous protein is achieved in the cytoplasm. In addition, the precursor fibrous protein may have a control peptide so as to enable localization of the precursor fibrous protein in certain compartments of the plant cell, e.g. ER, chloroplasts or vacuoles. Preferred precursor fibrous proteins are tropocollagen and tropoelastin as well as derivatives and fragments thereof, respectively. For the expression of a gene coding for a precursor fibrous protein it is possible to use conventional expression vectors for plant cells. They comprise regulatory elements, e.g. enhancer, promoter and termination sequences detected in plant cells. Examples thereof are CaMV 35S promoter and termination sequences (cf. Odell, J.T. et al., Nature 313 (1985), 810-812). The expression vectors may also contain selection markers, e.g. a neomycin or kanamycin resistance gene. In addition, the expression vectors may contain sequences which favor their introduction into plant cells. For example, the expression vectors may contain T-DNA of binary vectors, such as pSR 8-30 or pSR 8-35/1, when they shall be introduced into plants *via Agrobacterium tumefaciens* (cf. Düring, K. et al., Plant Journal 3 (1993), 587-598; Porsch, P. et al., Plant Molecular Biology 37 (1998), 581-585). Besides, the expression vectors can also be introduced into plant cells by means of processes for which they do not require any special sequences. Such processes are e.g. microinjection, electroporation, DNA transfer by means of polyethylene glycol, liposome fusion or particle gun.

The expression "plant cell" comprises plant cells of any kind and origin. It may refer to individual plant cells, freshly isolated or established as a cell line, or those present in

an aggregation. The latter is e.g. a plant or part thereof. Examples of plants are monocotyl plants, such as corn, rice, wheat, barley and sugarcane, and dicotyl plants, such as potato, tobacco, tomato, tea, coffee, brassicaceaen, particularly rape and cabbage, and leguminaes, particularly pea, phaseolus, vicia and soybean.

The expression "protein processing precursor fibrous protein" comprises any protein which can convert a precursor fibrous protein into the corresponding fibrous protein. The conversion can be made as usual, e.g. by cross-linkage or polymerization. Examples of such a protein are lysine oxidases. Also, proteinases may be concerned which, e.g. in the case of collagen, have been described. The lysine oxidases and proteinases, respectively, may be present as such and as derivatives or fragments thereof, respectively. The above statements made on a modified form of a fibrous protein apply correspondingly to them.

The expression "incubation of a precursor fibrous protein with a protein processing it" comprises any incubation of these proteins by which the precursor fibrous protein can be converted into the corresponding fibrous protein. The incubation may be made e.g. *in vitro*. For this purpose, it is favorable to incubate the expressed precursor fibrous protein in solution with the protein processing it. The incubation can also be carried out *in vivo*. For this purpose, it is favorable to express not only the precursor fibrous protein but also the protein processing it in a plant cell. Both proteins can be expressed in different plant cells which are then combined whereby the precursor fibrous protein is incubated with the protein processing it. The precursor fibrous protein and the protein processing it can also be expressed in the same plant cell. Thus, both proteins are automatically incubated in this plant cell. The above statements made on the expression of a precursor fibrous protein apply correspondingly to the expression of a protein processing a precursor fibrous protein.

A further subject matter of the present invention relates to a plant cell which expresses a precursor fibrous protein and a protein processing it. Also, a plant cell is preferred which expresses only the latter of these proteins. Regarding the expressions "plant cell", "precursor fibrous protein" and "protein processing precursor fibrous protein" reference is made to the above statements. In addition, the plant cell may be available in the form of a multiplication material.

Common methods can be used for the production of a plant cell according to the invention. In supplement to the above statements, the production of a plant according to the invention which expresses a precursor fibrous protein, e.g. tropoelastin, and a protein processing it, e.g. lysine oxidase, is described by way of example. In this connection, it is favorable to provide a cDNA coding for tropoelastin with CaMV 35S promoter and termination sequences and insert it in a binary vector, e.g. pSR 8-30 and pSR 8-35/1, respectively. The same can be done with a cDNA coding for a lysine oxidase. The resulting DNA molecules are used for transforming bacteria, e.g. E. coli S17-1 which are suitable for a transfer of the DNA molecules to *Agrobacterium tumefaciens*, e.g. GV 3101. For this purpose, E. coli S17-1 and *Agrobacterium tumefaciens* GV 3101 are mixed with each other and incubated overnight. Agrobacteria which have taken up the DNA molecules are selected by growth on carbenicillin-containing medium. They are then applied to cut-off potato plant leaves whose middle ribs were scratched several times and incubated in the dark for two days. Thereafter, the agrobacteria are removed and growth promoters are added to the potato plants, so that sprouts grow. They are cut off and used for cultivating new potato plants. The detection of the expression products tropoelastin and lysine oxidase and/or the resulting elastin is made by means of specific antibodies against these proteins. Reference is made to the below examples.

By means of the present invention it is possible to produce fibrous proteins in plant cells, particularly plants, in high

purity. Therefore, the fibrous proteins are suitable for the most varying applications. They are found e.g. in agriculture, chemistry, production of cosmetics and medicine. In the latter case, e.g. the use of fibrous proteins for transplants and wound closures has to be mentioned. In particular, the fibrous proteins distinguish themselves in that they are free from animal or human viruses and pathogens, respectively. Moreover, the fibrous proteins can be produced in huge amounts. This applies particularly when they are isolated from plants cultivated in fields. Thus, the present invention represents a great contribution to providing pharmaceutical preparations safely and in great amounts.

The invention is explained by the below examples.

Example 1: Production of elastin in potato plants

A cDNA is used for human elastin (cf. Fazio, M.J., Journal of Investigative Dermatology 91 (1988), 458-464). This cDNA is provided with an NcoI restriction site at its 5' end and with an XbaI restriction site at its 3' end by means of PCR. The resulting cDNA fragment is inserted in the vector pRT 100 which contains an expression cassette having CaMV 35S promoter and termination sequences (cf. Töpfer, R. et al., Nucleic Acids Research 15 (1987), 5890; Odell, J.T. et al., above). Following cleavage using HindIII, the expression cassette containing the elastin cDNA is isolated and inserted in the binary vector pSR 8-30 (cf. Düring, K. et al.; Porsch, P. et al., above). The expression vector pSR 8-30 elastin is obtained.

In addition, a cDNA for human lysine oxidase is used (cf. Hämäläinen, E.R., Genomics 11 (1991), 508-516). It is treated as described above and inserted in the binary vector pSR 8-30. The expression vector pSR 8-30 lysine oxidase is obtained.

The expression vectors pSR 8-30 elastin and pSR 8-30 lysine

oxidase are used for transforming E. coli S17-1. The transformants are mixed with *Agrobacterium tumefaciens* GV 3101 and incubated at 27°C overnight (cf. Koncz, C., Shell, J., Molecular and General Genetics 204 (1986), 383-396; Koncz, C. et al., Proc. Natl. Acad. Sci. U.S.A. 84 (1987), 131-135). Selection on carbenicillin is carried out, the bla gene necessary for this purpose being present in the above expression vectors. Selection clones of *Agrobacterium tumefaciens* are applied to cut-off leaves of potato plant cv. or named Désirée, whose middle ribs had been scratched several times and the plant is incubated in the dark at 20°C for 2 days. Thereafter, the agrobacteria are separated and growth promoters are added to the potato plant, so that sprouts form preferably. Moreover, non-transformed cells of the potato plant are killed by the addition of kanamycin to the plant medium. Rising sprouts are cut off and are allowed to grow roots on medium without plant growth substances but with kanamycin. The potato plants are further cultivated as usual.

The analysis of the expressed tropoelastin and lysine oxidase and/or the resulting elastin is achieved by antibodies in Western blot and ELISA, respectively, which are specific to the individual proteins. For this purpose, whole protein or the intercellular wash liquid of the potato plant is isolated and used in the corresponding detection methods.

It shows that tropoelastin and lysine oxidase can be expressed in plant cells, particularly in a plant. Moreover, it shows that by the incubation of lysine oxidase with the tropoelastin the latter is converted into elastin which can be isolated in pure form.

Example 2: Production of collagen in potato plants

cDNAs are used which code for the subunits α1 and α2 of human tropocollagen (cf. Chu, M.L. et al., Journal of Biological Chemistry 260 (1985), 2315-2320; Dickson L.A. et al., Nucleic

Acids Res. 13 (1985), 3427-3438). Furthermore, cDNAs are used which code for human lysine oxidase, human procollagen C proteinase and procollagen N proteinase, respectively, from bovine animals (cf. Hämäläinen, E.R. et al., above; Li, S.W. et al., Proc. Natl. Acad. Sci U.S.A. 93 (1996), 5127-5130; Colige, A. et al., Proc. Natl. Acad. Sci. U.S.A. 94 (1997), 2374-2379).

These DNAs are treated as described in Example 1 and inserted in the pSR 8-30 vector. The expression vectors pSR 8-30 tropocollagen α1, pSR 8-30 tropocollagen α2, pSR 8-30 lysine oxidase, pSR 8-30 C proteinase and pSR 8-30 N proteinase are obtained. The procedure is continued as described in Example 1.

It shows that tropocollagen and proteins processing it can be expressed in plant cells, particularly in a plant. In addition, it shows that collagen having a high degree of purity can be obtained.

Claims

1. A process for the production of a fibrous protein, comprising the following steps:

(a) expression of a precursor fibrous protein in a plant cell, and

(b) incubation of the precursor fibrous protein with a protein processing it.

2. The process according to claim 1, wherein the processing protein is expressed in a plant cell.

3. The process according to claim 2, wherein the precursor fibrous protein and the protein processing it are expressed in different plant cells.

4. The process according to claim 2, wherein the precursor fibrous protein and the protein processing it are expressed in the same plant cell.

5. The process according to any one of claims 1 to 4, wherein the plant cell is available in the form of a plant.

6. The process according to any one of claims 1 to 5, wherein the precursor fibrous protein is a procollagen or a derivative and fragment thereof, respectively.

7. The process according to any one of claims 1 to 5, wherein the precursor fibrous protein is a tropoelastin or a derivative and fragment thereof, respectively.

8. The process according to any one of claims 1 to 6, wherein the fibrous protein is a collagen or a derivative and fragment thereof, respectively.

9. The process according to any one of claims 1 to 5 and 7, wherein the fibrous protein is an elastin or a derivative and fragment thereof, respectively.

10. The process according to any one of claims 1 to 9, wherein the protein processing precursor fibrous protein is a lysine oxidase.

11. A plant cell, expressing a precursor fibrous protein and a protein processing it.

12. The plant cell according to claim 11, wherein the plant cell is available in the form of a multiplication material.

13. The plant cell according to claim 11, wherein the plant cell is present in the form of a plant.

14. The plant cell, expressing a protein processing precursor fibrous protein.

15. The plant cell according to claim 14, wherein the plant cell is available in the form of a multiplication material.

16. The plant cell according to claim 14, wherein the plant cell is available in the form of a plant.

17. Use of the plant cell according to any one of claims 11 to 16 for the production of a fibrous protein.

18. The fibrous protein, produced according to the process as defined in any one of claims 1 to 10.

19. The fibrous protein according to claim 18, wherein the fibrous protein is a collagen or a derivative and fragment thereof, respectively.

20. The fibrous protein according to claim 18, wherein the fibrous protein is an elastin or a derivative and fragment thereof, respectively.

Abstract of the Disclosure

The present invention relates to a process for the production of a fibrous protein, comprising the following steps:

(a) expression of a precursor fibrous protein in a plant cell, and

(b) incubation of the precursor fibrous protein with a protein processing it.

Furthermore, this invention concerns plant cells usable for this purpose and fibrous proteins obtained by this process.

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(54) Title: FIBROUS PROTEINS AND THE PRODUCTION THEREOF

(54) Bezeichnung: FASERPROTEINE UND IHRE HERSTELLUNG

(57) Abstract

The present invention relates to a method for the production of a fibrous protein, comprising the following steps: (a) expression of a precursor fibrous protein in a plant cell and (b) incubation of the precursor fibrous protein with a protein processing the latter. The invention also relates to the plant cells used for this purpose and to the fibrous proteins produced according to the inventive method.

(57) Zusammenfassung

Die vorliegende Erfindung betrifft ein Verfahren zur Herstellung eines Faserproteins, umfassend die folgenden Verfahrensschritte: (a) Expression eines Vorläufer–Faserproteins in einer Pflanzenzelle; und (b) Inkubation des Vorläufer–Faserproteins mit einem es prozessierenden Protein. Ferner betrifft die Erfindung hierfür verwendbare Pflanzenzellen und durch das Verfahren erhaltene Faserproteine.

DECLARATION FOR NON-PROVISIONAL PATENT APPLICATION*

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below at 201 et seq. beneath my name.

I believe I am the original, first and sole inventor if only one name is listed at 201 below, or an original, first and joint inventor if plural names are listed at 201 et seq. below, of the subject matter which is claimed and for which a patent is sought on the invention entitled

FIBROUS PROTEINS AND THE PRODUCTION THEREOF

and for which a patent application:
☒ is attached hereto and includes amendment(s) filed on *(if applicable)*
☐ was filed in the United States on as Application No. *(for declaration not accompanying application)*
with amendment(s) filed on *(if applicable)*
☒ was filed as PCT International Application No. PCT/DE99/02359 on 3 August 1999.

I hereby state that I have reviewed and understand the contents of the above identified application, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

EARLIEST FOREIGN APPLICATION(S), IF ANY, FILED PRIOR TO THE FILING DATE OF THE APPLICATION			
APPLICATION NUMBER	COUNTRY	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
198 34 909.2	Germany	3 August 1998	YES ☒ NO ☐
			YES ☐ NO ☐
			YES ☐ NO ☐

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below.

PROVISIONAL APPLICATION NUMBER	FILING DATE

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information known to me which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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